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Antibodies Generated against Conserved Antigens Expressed by Bacteria and Allergen-Bearing Fungi Suppress Airway Disease

Nicholas W. Kin,[1] Emily K. Stefanov,[1] Brian L. P. Dizon, and John F. Kearney

There has been a sharp rise in allergic asthma and asthma-related deaths in the developed world, in contrast to many childhood illnesses that have been reduced or eliminated. The hygiene hypothesis proposes that excessively sanitary conditions early in life result in autoimmune and allergic phenomena because of a failure of the immune system to receive proper microbial stimulation during development. We demonstrate that Abs generated against conserved bacterial polysaccharides are reactive with and dampen the immune response against chitin and *Aspergillus fumigatus*. A reduction in Ag uptake, cell influx, cell activation, and cytokine production occurred in the presence of anti-polysaccharide Abs, resulting in a striking decrease in the severity of allergic airway disease in mice. Overall, our results suggest that Ag exposure—derived from environmental sources, self-antigens, or vaccination—during the neonatal period has dramatic effects on the adult Ab response and modifies the development of allergic airway disease.   *The Journal of Immunology*, 2012, 189: 2246–2256.

The incidence of asthma, an increasingly significant public health issue with a clear association with immune allergies, is more prevalent in Western-style societies. The hygiene hypothesis attributes this increase to reduced stimulation of the immune system by microorganisms, due in part to the increased sanitary conditions early in life (1). Perinatal and early childhood periods are considered critical for development of a normal Th1/Th2 balance of effector CD4 T cells, and it is thought that the absence of appropriate microbial exposure during this period leads to a shift from a Th1 to a Th2 CD4 T cell cytokine profile. This shift is accompanied by increased allergic phenomena, including production of allergen-specific IgE Abs that exacerbate asthma pathology. However, the similar rise in autoimmune diseases during this period cannot be explained through the Th1/Th2 paradigm (2). In addition, identification of specific infectious agents or assessment of the underlying immunological mechanisms responsible for these increases have yielded conflicting results (3). We propose an adjunct hypothesis that Abs may contribute to the mechanism of protection proposed by the hygiene hypothesis.

Allergens involved in asthma and other allergic diseases are a highly diverse group of molecules, and it is becoming increasingly clear that their ability to induce allergies resides in their presentation as "cargo" associated with innate immune-activating components (4, 5). One such immune-activating molecule that has attracted recent attention is chitin, a naturally occurring β-1,4-linked *N*-acetyl-glucosamine (GlcNAc) homopolymer. As the second most abundant biopolymer on Earth, chitin is ubiquitously associated with a multitude of organisms implicated in human allergies including fungi, molds, crustaceans, insects, and parasites. Furthermore, purified chitin particles exert size-dependent effects on innate and adaptive immunity, leading to the proposal that chitin and chitinases play a role in pulmonary inflammatory and allergic responses (6, 7). However, the physical nature of purified commercially available chitin used by most investigators bears little resemblance to organism-associated chitin. In its natural unpurified state, chitin is covalently linked to proteins and other glucans, as well as other organic and inorganic molecules, particularly in fungi (8). Therefore, chitin's role in asthma and allergic diseases is best studied in the context of its naturally occurring state within the environment.

Aspergillus fumigatus, a ubiquitous pathogenic fungus, and the other chitin-containing organisms mentioned above have been shown to be linked to allergic airway diseases dependent on a variety of geographical and environmental associations (9, 10). *A. fumigatus* expresses an array of highly conserved cell wall-associated polysaccharides during its life cycle, including chitin (7–15%), α-1,3 glucans (35–46%), and β-1,3 glucans (20–35%) (11). There is a variety of innate receptors for these fungal cell wall polysaccharides including the mannose receptor (CD206) (12) and TLR2 (13) for chitin and dectin-1 (14, 15) and CD36 (16) for β-glucans, to name a few (extensively reviewed in Ref. 17). Interactions of these cell wall structures and innate receptors are involved in a wide range of inflammatory and allergic responses induced by these organisms. Notably, fungi share similar polysaccharide epitopes with commensal and pathogenic bacteria. For example, *Enterobacter cloacae* (18, 19) *Streptococcus pyogenes* (group A streptococci; GAS), and *Streptococcus agalactiae* (group 1b *Streptococcus*) (20) express α-1,3 glucans, GlcNAc, and sialyllacto-*N*-tetraose, respectively, all of which induce polysaccharide-specific

Abs after immunization/infection. We have taken all these findings together and developed an adjunct hypothesis to the prevailing idea that infections early in life may modify the Th1/Th2 balance and prevent the development of allergies/asthma. We propose that natural Abs generated against conserved bacterial polysaccharides alter the interactions between allergen-bearing microorganisms and innate receptors in the lung microenvironment and dampen susceptibility to asthma and other allergy-associated diseases.

Throughout infancy, childhood, and adolescence, the immune system is in a constant state of development and maturation, and these processes are susceptible to extrinsic influences from the environment. The discovery of genes associated with asthma is in its infancy, but it is unlikely that a single mechanism will be found responsible for induction of this complex disease. With allergic asthma often developing early in childhood, we propose that the highly plastic clonal B cell repertoire is altered during a critical time in B cell development by early exposure to environmental Ags. Such alterations in B cell clonal frequencies and the BCR repertoire produce long-lasting effects on adult natural Ab levels and thus Ab-mediated protection or susceptibility to allergen-induced airway disease. In the current study, we demonstrate that Abs generated against conserved bacterial polysaccharides are reactive with and dampen inflammatory and allergic responses to *A. fumigatus* after intratracheal (i.t.) challenge. Using a chronic *A. fumigatus* i.t. sensitization protocol, we show that passive transfer of germline gene-encoded Abs to adults or bacterial immunization of neonates inhibits the influx and activation of innate and adaptive immune cells in both the lung tissue and alveolar spaces. The inhibition of cellular influx was associated with reduced production of asthma-associated cytokines and chemokines. Furthermore, the presence of these bacteria- and fungi-reactive Abs reduced the uptake and activation of chitin and *A. fumigatus* by macrophages and dendritic cells. The current studies reveal that neonatal Ag exposure induces alterations in the B cell clonal frequency and/or repertoire, which has long-lasting effects on the adult Ab repertoire and thus provides a level of resistance to the development of *A. fumigatus*-induced allergic airway disease (AAD) in mice.

Materials and Methods

Animals

C57BL/6 mice were purchased from The Jackson Laboratory and maintained under specific pathogen-free conditions using approved animal protocols of the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Preparation of fungus

A. fumigatus isolate 13073 (American Type Culture Collection, Manassas, VA) was maintained on potato dextrose agar for 5 d at 37°C. Conidia were harvested by washing the culture flask with 50 ml sterile PBS. Conidia were filtered through a sterile 40-μm nylon membrane to remove hyphal fragments, enumerated on a hemocytometer and stored at 4°C in distilled water until use. *A. fumigatus* was germinated in DMEM plus 10% FCS at 37°C for various periods of time prior to staining and analysis by flow cytometry.

CD4 T cell cytokine staining

For determination of in vivo IL-4 production, total lung lymphocytes were isolated by collagenase digestion 24 h after final *A. fumigatus* challenge. Lymphocytes (10^6) were cultured in a 96-well plate in RPMI 1640 plus 10% FCS and brefeldin A (BD Biosciences) for 6 h in the presence or absence of 5 μg/ml plate-bound anti-CD3 and anti-CD28 (eBioscience). Cells were then stained with anti-CD4, fixed in Cytofix/Cytoperm (BD Biosciences), and stained for IL-4 or isotype control in Permwash (BD Biosciences).

ELISPOT analysis

The total numbers of GlcNAc-specific Ab-secreting cells (ASCs) in the spleen and bone marrow were determined as previously described in Ref. 21 using 96-well EIA/RIA plates (Costar, Corning, NY) coated with PGN-GAC overnight at 4°C at a concentration of 2 μg/ml. Bone marrow or splenic cells (10×10^6–15×10^6) from individual C57BL/6 mice neonatally immunized with GAS on day 3 or day 14 of life and sacrificed at 8 wk of age were added to the first well and then diluted 5-fold in RPMI 1640 with 5% FCS to generate quantifiable spots.

Histology

Lungs were fixed in 4% PFA, dehydrated by sequentially increasing concentrations of ethanol, incubated in xylene, and embedded in paraffin. Four-micrometer sections of lung were cut, rehydrated, and stained with H&E or periodic acid–Schiff (PAS) and Alcian blue 8GX (Sigma-Aldrich). Sections were then dehydrated, incubated in xylene, and mounted in Permount (Fisher Scientific). Tissue sections were viewed as previously described (22).

Immunofluorescence

A. fumigatus conidia were bound to poly-L-lysine–coated glass slides and were incubated for 0, 4, 8, and 11 h in RPMI 1640 plus 2% FCS. The slides were stained with SMB19, GAC39, and 1-21 mAbs and viewed with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes and phase contrast.

Flow cytometry

Flow cytometry was performed as described previously (23). FITC-labeled anti-B220, anti-IgE, PE-labeled anti-Ly6G, anti-IL-4, anti-SiglecF, anti-CD44, Pacific blue-labeled anti-CD4, anti–IFN-γ, anti-B220, allophycocyanin-labeled anti-CD117, and anti-CD8 Abs were purchased from BD Pharmingen; goat anti-mouse IgM was purchased from Southern Biotechnology Associates (Birmingham, AL). FcR blocker Ab93, HGAC39 and HGAC78, 1-21, and SMB19 mAbs were generated and/or maintained in our laboratory. All FACS analyses were performed on a FACSCalibur (BD Biosciences) or LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Immunization

Three-day-old and 14-d-old mice were immunized i.p. or s.c. with 5×10^7 heat-killed, pepsin-treated *S. pyogenes* (strain J174A). Sera were collected at 8 wk (day 0) before reimmunization with 1×10^8 *S. pyogenes* and again on day 7 after reimmunization and stored at −80°C until use.

ELISA

GlcNAc-specific IgM and total IgE levels were determined by ELISA as previously described (24) using plates coated with rat anti-mouse IgE (Southern Biotechnology Associates) or GAC-PGN (Lee Labs) and detected with alkaline phosphatase-conjugated goat anti-mouse IgE (Southern Biotechnology Associates) or goat anti-mouse IgM (Southern Biotechnology Associates). Standard curves were prepared using known quantities of mouse IgE (Southern Biotechnology Associates) or GAC78, a GlcNAc-specific IgM purified in our laboratory. *p*-Nitrophenyl phosphate (Sigma-Aldrich) was added, and color development was determined on a SPECTROstar Omega reader (BMG Labtech) at 405 nm.

A. fumigatus-*specific ELISAs*

A. fumigatus-specific Abs in the bronchoalveolar lavage (BAL) fluid and serum were measured by coating Costar 96-well plates (Fisher Scientific) with a 1:100 dilution of *A. fumigatus* allergenic extract (Hollister Stier, Spokane, WA). Abs were detected with alkaline phosphatase-conjugated goat anti-mouse Ig (Southern Biotechnology Associates) and addition of *p*-nitrophenyl phosphate (Sigma-Aldrich). OD was measured using a SPECTROstar Omega reader (BMG Labtech) at 405 nm. Sera and BAL fluid from naive and PBS-treated mice were used as controls.

RT-PCR

RT-PCR was performed as previously described (21). Briefly, total RNA was isolated using TRIzol according to the manufacturer's instructions (Invitrogen). Total RNA was quantified by NanoDrop analysis (Thermo Scientific), and cDNA was generated using Omniscript RT-PCR kit (Qiagen).

In vivo i.t. challenge and lung and BAL fluid analysis

Mice were anesthetized using 3–5% isoflurane, and 100 μg purified chitin or 5×10^5 live *A. fumigatus* conidia was administered i.t. in a volume of 50 μl and in the presence or absence of polysaccharide-binding Abs. Mice were immobilized on a slanted/vertical board using a suture string looped around their upper incisors, with taped tail to help support body weight,

and the tongue was extended from the oral cavity using blunt end forceps. Liquid (50 μl) was pipetted in the oral cavity, and the nares were manually plugged using fingers to force the mouse to inhale the liquid. For an AAD model, *A. fumigatus* conidia were administered Mondays and Fridays for eight consecutive weeks. After a week off this regimen, mice were challenged i.t. with 5×10^5 conidia. One to three days after challenge, mice were sacrificed, and total BAL fluid and lung cells were analyzed by flow cytometry, ELISA, and cytokine analysis. BAL cells were enumerated using a hemocytometer and trypan blue dye exclusion. Cells were isolated from the lung using collagenase digestion as described previously (25).

Chitin preparations

Chitin particles from crab shells (Sigma) were prepared using a protocol published previously (12). Chitin was labeled with 488 and 647 Alexa fluorochromes according to the manufacturer's instructions (Molecular Probes/Invitrogen Life Technologies). Chitin contained <0.1 U/ml endotoxin as determined by Etoxate (Sigma), a *Limulus* lysate assay with a detection level of 0.1 U/ml.

Anti-A. fumigatus Abs

All anti–α-1,3 glucan hybridomas were generated in our laboratory (18, 19). Hybridomas producing SMB-19 (26), HGAC78 (27), and HGAC39 (28) have been previously described. IgG3 Ab was purified from the supernatant by absorption to protein G–Sepharose affinity columns from Amersham Biosciences, and IgM Abs were purified on Sepharose-6B columns coupled with RS3.1 anti-IgMa mAb. Purified Abs were labeled with Alexa fluorochromes according to instructions (Molecular Probes/Invitrogen Life Technologies). Abs were determined to have <0.1 U/ml endotoxin activity using Etoxate (Sigma). The "Antibody Combo" contains HGAC78, SMB-19, and an anti–α-1,3 glucan IgM.

BAL fluid cytokine analysis

The first milliliter of fluid extracted from the BAL of sacrificed mice was used to measure the levels of 23 cytokine and chemokine proteins using the Bio-Plex Multiplex Suspension Cytokine Array (Bio-Rad Laboratories) according to the manufacturer's instructions. The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories).

In vitro cell cultures

Bone marrow-derived macrophages (BMDMs) were generated as described previously (29). A459 epithelial cells or BMDMs were seeded at 500,000 cells/well in 6-well plates in DMEM plus 10% FCS and 100 μM 2-mercaptoethanol. The cells were incubated with either unlabeled or Alexa 647-labeled chitin or Alexa 488-labeled *A. fumigatus* in the presence or absence of 10 μg/ml *A. fumigatus*-binding Abs or isotype controls for 2–24 h at 37°C. In *A. fumigatus, Bacillus anthracis* dual uptake studies, 10^6 Alexa 647-labeled *B. anthracis* spores (Sterne strain) were added to cultures containing 5×10^5 Alexa 488-labeled *A. fumigatus* conidia. Uptake of spores and conidia were analyzed by flow cytometry or RT-PCR.

Statistics

Statistical comparisons were performed using Prism 4.0 software (GraphPad). Data with three or more groups were analyzed by a one-way ANOVA test followed by post hoc analysis, whereas data with two groups were analyzed by a two-tailed unpaired *t* test to determine whether overall statistically significant differences existed. Statistically significant results are indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Abs generated against conserved bacterial polysaccharides bind chitin and A. fumigatus

mAbs mostly of IgM, IgG3, and IgA isotypes were isolated from mice immunized with *E. cloacae* (18, 19), *S. pyogenes* (30), and *S. agalactiae* (26). Because immunization of mice with these bacteria generates large amounts of serum Abs against their respective cell wall polysaccharides (PSs), we investigated whether Abs generated by bacterial immunization bound fungi. Two different representative IgM and IgG3 anti-GlcNAc mAbs generated from GAS-immunized mice bound purified chitin particles <10 μm in size (Fig. 1A). In addition, live resting or 18-h-germinated *A. fumigatus* conidia were stained with anti–sialyllacto-*N*-tetraose (SMB19), anti-GlcNAc (GAC39), and anti–α-1,3 glucan (1-21) but not by an

isotype control anti-human myelomonocytic acid Ab (Fig. 1B). Furthermore, the expression of these Ags increased with time of germination and became maximal during the hyphal stage (Fig. 1C). These studies show that multiple mAbs generated against commensal and pathogenic bacteria also react with ubiquitous cell wall components of *A. fumigatus* and other fungi (data not shown).

Anti-GlcNAc Abs dampen chitin-induced cellular recruitment and activation in the lung

As a proof of principle, we first examined whether bacteria-induced chitin-reactive Abs could alter the immune response to defined chitin particles in the lung. Purified chitin particles have been shown to induce the recruitment of asthma/allergy-associated cell types (6). Administration of IgM anti-GlcNAc Ab, via either the i.t. or i.v. route, inhibited chitin-induced recruitment of neutrophils into the BAL fluid by >50% compared with the isotype control Ab (Fig. 2B, gating scheme is shown in Fig. 2A and Supplemental Fig. 1). To investigate potential mechanisms through which the anti-GlcNAc Ab inhibits cellular recruitment, cytokine levels in BAL fluid were measured 24 h after chitin administration. The production of the proinflammatory cytokines KC, RANTES, MIP-1α, and TNF-α was inhibited by ∼50% after anti-GlcNAc administration (Fig. 2C). These cytokines and chemokines are produced by various cell types in the lung including vascular endothelium, epithelial cells, macrophages, dendritic cells, and T lymphocytes. Cells and mRNA were obtained from lung tissue digests and, similar to our findings in the BAL fluid, the presence of anti-GlcNAc Ab partially inhibited the recruitment of all cell types into the lung tissue including neutrophils and CD4 T cells (Fig. 2D). Additionally, reduced mRNA transcript levels of CCL17 and CCL22 were observed in the lungs in the presence of anti-GlcNAc Abs (Fig. 2E). Taken together, these data show that the presence of anti-GlcNAc Ab greatly reduces chitin-induced inflammation and influx of cells into the lung tissue and alveolar spaces, thus dampening the overall immune response to chitin.
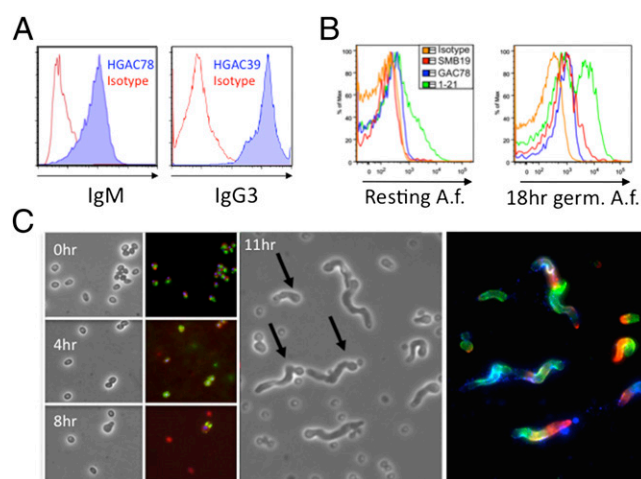

FIGURE 1. mAbs generated against conserved bacterial PSs bind chitin and *A. fumigatus*. (**A**) Purified chitin particles (<10 μm) were incubated with anti-GlcNAc IgM (HGAC78, blue), IgG3 (HGAC39, blue), or isotype control (Isotype, red) Abs and analyzed by flow cytometry. (**B**) Live resting or 18-h-germinated *A. fumigatus* conidia were incubated with an IgM isotype control (Isotype, orange), anti–sialyllacto-*N*-tetraose (SMB19 IgM, red), anti-GlcNAc (HGAC39 IgG3, blue), or anti–α-1,3 glucan (1-21 IgM, green) Abs and analyzed by flow cytometry. (**C**) *A. fumigatus* conidia bound, poly-L-lysine–coated glass slides were incubated for 0, 4, 8, and 11 h in RPMI 1640 plus 2% FCS. Columns 1 and 3 show a field (original magnification ×1000) under phase contrast with germinated conidia (arrow), and columns 2 and 4 show staining with SMB19 (red), GAC39 (blue), and 1-21 (green).
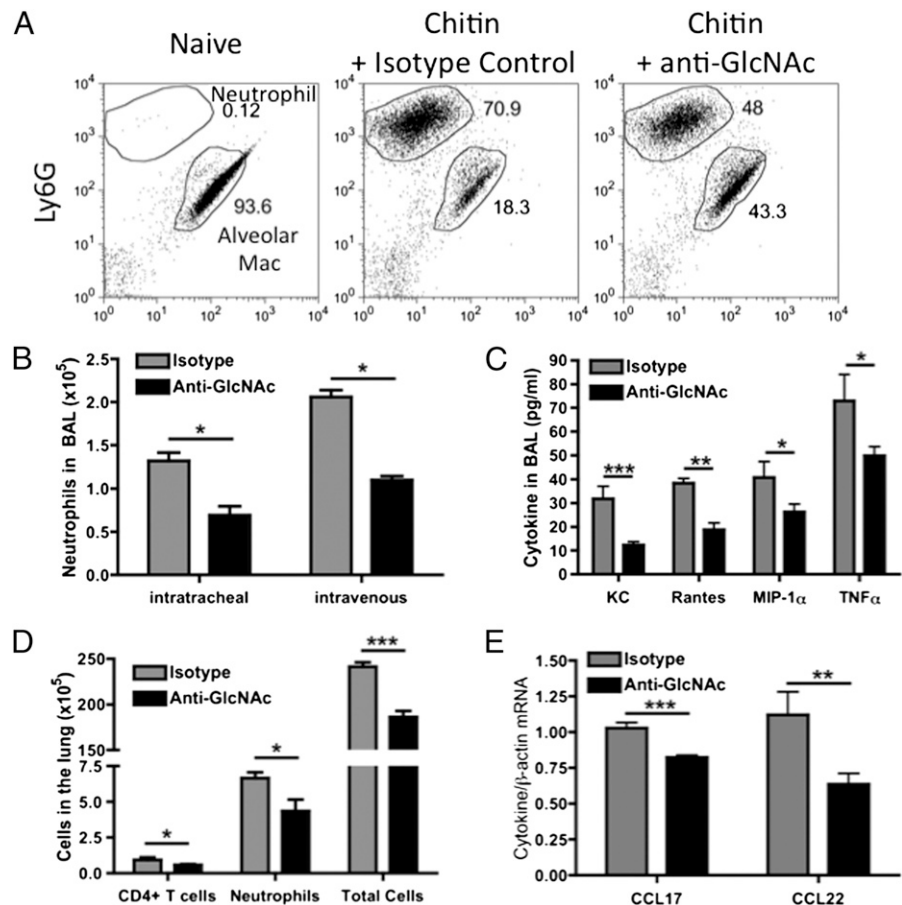
FIGURE 2. Anti-GlcNAc Ab dampens chitin-induced cellular recruitment and activation in the lung. C57BL/6 mice, treated i.v. with 50 μg IgM isotype control or anti-GlcNAc Abs [i.v. and i.t. in (**B**)], were challenged i.t. with 100 μg chitin particles. BAL fluid was collected 24 h after chitin challenge, and the total numbers of neutrophils (Ly6G$^+$) and alveolar macrophages (determined by their autofluorescence) [gating scheme shown in (**A**)] were enumerated by flow cytometry (**B**). (**C**) Cytokine expression was measured by a Bio-Plex cytokine array. (**D**) Cells collected from collagenase-treated lungs and total cell numbers, neutrophils, and CD4$^+$ T cells were calculated. (**E**) Total RNA was collected from lung tissue-derived cells and analyzed by RT-PCR for CCL17 and CCL22 expression. Values represent the mean ± SEM from three independent experiments with three to five mice per group. Data were analyzed by a two-tailed unpaired t test. See also Supplemental Fig. 1. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Anti-GlcNAc Abs decrease the uptake of chitin particles in vitro and in vivo

Although the data in the previously cited figures clearly demonstrate that anti-GlcNAc Ab binds chitin and dampens the immune response to lung-administered chitin, the mechanism by which this occurs is unclear. To determine whether anti-GlcNAc Abs were affecting the binding or cellular uptake of chitin, Alexa 647-labeled chitin was used to quantify the number of chitin-positive cells in an uptake assay (gating scheme shown in Fig. 3A). BMDMs and A549 epithelial cells were cultured with Alexa 647-labeled chitin, in the presence or absence of an IgM isotype control or anti-GlcNAc Ab, and cellular binding and uptake of *A. fumigatus* was measured by flow cytometry. The anti-GlcNAc Ab decreased the uptake of chitin particles by both BMDMs (Fig. 3B) and A549 cells (Fig. 3C). We next treated C57BL/6 mice i.t. with an IgM isotype control or anti-GlcNAc Ab and subsequently challenged i.t. with Alexa 647-labeled chitin particles. BAL was collected at 0, 1, 2, and 3 h. Similar to our the findings in vitro, alveolar macrophages (Fig. 3D) and dendritic cells (Fig. 3E) bound or internalized less chitin in the presence of anti-GlcNAc Ab, as shown by flow cytometry. Taken together, the data show that anti-GlcNAc Ab decreases the uptake of chitin particles both in vitro and in vivo, suggesting that it interferes with the cellular recognition mechanisms for chitin.

Anti-A. fumigatus Abs dampen the allergic airway response induced by A. fumigatus

To determine further the effects of *A. fumigatus*-binding Abs, we adapted a low-dose airway sensitization model (31) using live *A. fumigatus* conidia more closely to mimic the physiological process of allergen sensitization in humans. Eight-week-old mice

were sensitized by i.t. instillation of a noninflammatory, low-dose *A. fumigatus* for 8 wk. This dose was previously established to have no effect, as a single dose, on cell infiltration or recruitment into the lung (data not shown). Mice were then rested for 1 wk and rechallenged i.t. with *A. fumigatus* (protocol depicted in Fig. 4A). This regimen of chronic *A. fumigatus* sensitization and rechallenge reproducibly induces a strong allergic response, with increases in total BAL cells (>10-fold), eosinophils (>12-fold), neutrophils (>5-fold), mast cells (>10-fold), and total serum IgE (>4-fold) (data not shown). Using this model system, we sought to determine if anti-GlcNAc and other *A. fumigatus*-binding anti-PS mAbs would dampen the induction of an allergic airway response to *A. fumigatus*. Mice were treated with anti-GlcNAc, anti-α-1,3 glucan, and anti–sialyllacto-*N*-tetraose Abs singly or in combination i.v. 2 h prior to the final i.t. challenge with *A. fumigatus*. The mice were sacrificed 3 d later, and the BAL fluid and total lung-associated leukocytes were analyzed by flow cytometry (gating scheme shown in Supplemental Fig. 1). Passive administration of anti-GlcNAc or the Ab combination decreased the total number of neutrophils and eosinophils present in the BAL fluid by at least 50% (Fig. 4B). We found that these Abs also reduced the total number of neutrophils (Fig. 4C), eosinophils (Fig. 4C), CD8 T cells (Fig. 4E), and the CD44 expression on CD4 T cells (as shown by mean fluorescence intensity; Fig. 4D) within the lung tissue by ~50%. Individual Abs of both IgM and IgG3 isotypes exhibited a hierarchy of ability to protect, with anti-GlcNAc and anti–sialyllacto-*N*-tetraose Abs inhibiting neutrophil influx equally, whereas the anti-α1,3 glucan Ab was less effective (data not shown). These data clearly demonstrate the ability of these anti-PS Abs to alter the allergic response to *A. fumigatus* in the lung.
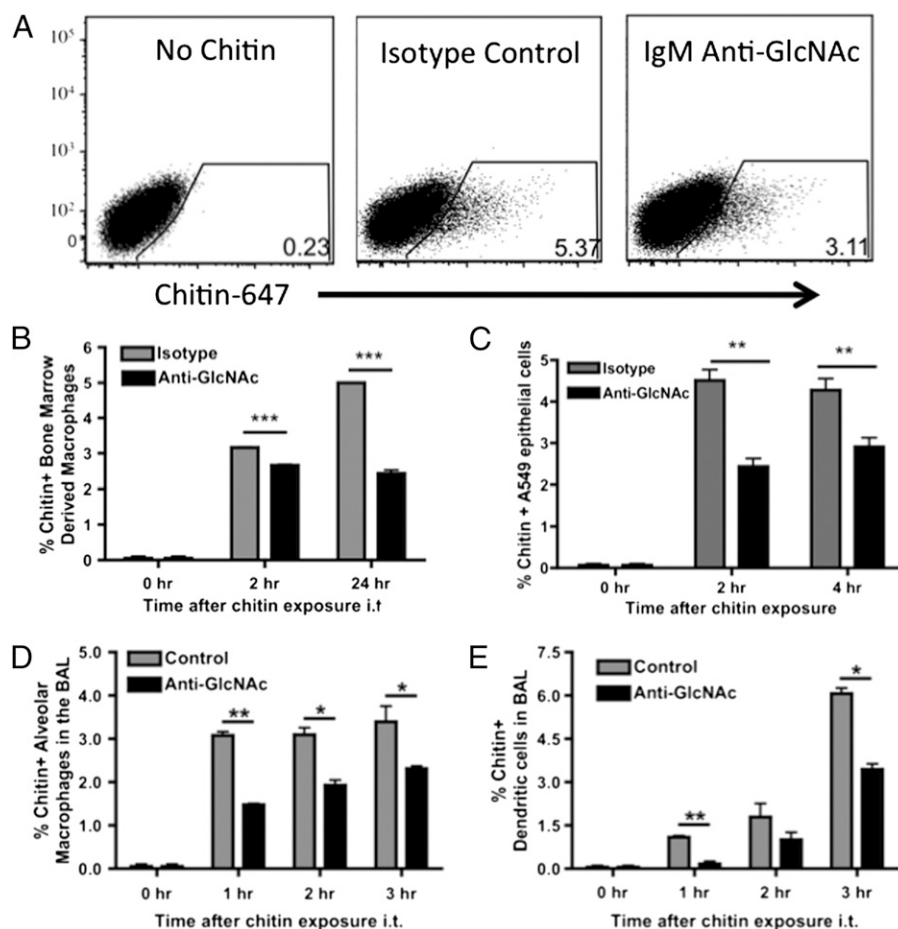
FIGURE 3. Anti-GlcNAc Ab decreases the uptake of chitin particles in vitro and in vivo. (**A**) Representative gating scheme for determining chitin-positive cells. (**B**) BMDMs and (**C**) A549 epithelial cells were cultured with 100 μg Alexa 647-labeled chitin, in the presence or absence of an IgM isotype control or anti-GlcNAc Ab, and chitin uptake was determined by flow cytometry. C57BL/6 mice, treated i.t. with an IgM isotype control or anti-GlcNAc Abs, were challenged i.t. with 100 μg Alexa 647-labeled chitin particles, and BAL fluid was collected at 0, 1, 2, and 3 h. Chitin-positive (**D**) alveolar macrophages and (**E**) dendritic cells were detected by flow cytometry. Data represent the mean ± SEM from three independent experiments with 6 wells or three to five mice per group. Data were analyzed by a two-tailed unpaired t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

*Anti-*A. fumigatus *Abs decrease the uptake of* A. fumigatus *in vitro and in vivo*

The previously cited figures clearly show that anti-PS Abs bind to and dampen the immune response against *A. fumigatus* conidia, and our studies with purified chitin and anti-GlcNAc Abs suggest that the Abs can block interactions between chitin and its cellular receptors. We examined if the *A. fumigatus*-binding anti-PS Abs could inhibit uptake and/or binding of *A. fumigatus* conidia to cells. BMDMs and A549 epithelial cells were cultured with Alexa 488-labeled *A. fumigatus* conidia, in the presence of either anti-PS Abs or an IgM isotype control. Binding or cellular uptake was then measured using flow cytometry (gating scheme shown in Fig. 5A). BMDMs and A549 bound or internalized less *A. fumigatus* in the presence of anti-PS Abs at 2 and 4 h (Fig. 5B, 5C). To determine whether this finding was specific to our Abs binding *A. fumigatus* or a cell-intrinsic inhibition initiated by Ag–Ab complexes, we measured cell uptake of simultaneously administered Alexa 488-labeled *A. fumigatus* conidia and Alexa 647-labeled *B. anthracis* spores in the presence of anti-PS or isotype control Abs in BMDMs and A549 cells. Anti-PS Abs bound and decreased the uptake of *A. fumigatus*, whereas the uptake of *B. anthracis* was unchanged (Supplemental Fig. 2A, 2B). To measure *A. fumigatus* uptake in vivo, C57BL/6 mice were treated with either an IgM isotype control or an anti-PS Ab combination, challenged i.t. with Alexa 488-labeled *A. fumigatus* conidia, and BAL fluid analyzed at 0, 2, and 4 h. The presence of anti-PS Abs decreased the percentage of *A. fumigatus*-positive alveolar macrophages (Fig. 5D) and dendritic cells in the BAL fluid (Fig. 5E). These results show collectively that treatment with anti-PS Abs decreased the binding

or internalization of *A. fumigatus* by APCs in vitro and in vivo, similar to our findings with chitin.

Neonatal GAS immunization primes the adult Ab response and dampens the immune response to chitin

We have hypothesized that the lack of microbial stimulation early in life and its effect on B cells and Ab production is partially responsible for the increased development of AAD. The previous experiments show that passive administration of purified mAbs, by i.v. or i.t. routes, reduces the allergic inflammatory response in previously sensitized adult mice. To examine how neonatal Ag exposure can generate Ab repertoires that dampen the development of AAD, we immunized C57BL/6 mice neonatally with GAS at 3 or 14 d after birth. Serum levels of GlcNAc-specific IgM in these mice at 8 wk of age were ∼9 and 50 μg/ml, respectively, compared with 5 μg/ml in PBS-treated mice, showing the initial neonatal immunization had a lasting effect. Upon rechallenge with GAS, these day 3 and day 14 neonatally primed animals produced a 4- and 10-fold increase, respectively, in GlcNAc-specific IgM compared with the PBS-treated animals (Fig. 6A). Additionally, the levels in anti-GlcNAC IgM in the serum of day 3-immunized mice increased 310-fold over preimmune serum levels compared with a 100-fold increase in PBS-treated mice. ELISPOT analysis demonstrated an increase in GlcNAc-specific IgM splenic ASCs in the GAS-primed mice compared with PBS-treated mice (Fig. 6B). Further analysis showed increased levels of GlcNAc-specific IgM in the BAL fluid (Fig. 6C), which was associated with a decreased influx of neutrophils in response to chitin (Fig. 6D). To confirm that the priming of the anti-GlcNAc Ab response was not a phenomenon associated only with i.p. neonatal GAS immunization, we s.c. immunized

FIGURE 4. Anti-*A. fumigatus* Abs dampen the AAD induced by *A. fumigatus*. (**A**) C57BL/6 mice were subjected to 5×10^5 live *A. fumigatus* conidia, starting at 8 wk of age, administered i.t. twice a week for 8 wk. After 1 wk without challenge, AAD was elicited using the same dose of *A. fumigatus* conidia, in the presence or absence of an isotype control, anti-GlcNAc Ab, or a combination of anti-GlcNAC, anti–sialyllactose-*N*-tetraose, anti–α-1,3 glucan, and lungs were analyzed 3 d later. (**B**) BAL fluid and (**C**) cellular lung digests were collected and analyzed for neutrophil and eosinophil cell infiltration using flow cytometry. The level of (**D**) CD44 expression by CD4$^+$ T cells and (**E**) total CD8$^+$ T cells was measured by flow cytometry. Data represent the mean ± SEM from three independent experiments with three to five mice per group. Data were analyzed by a two-tailed unpaired *t* test. See also Supplemental Fig. 1. $*p < 0.05$, $**p < 0.01$.
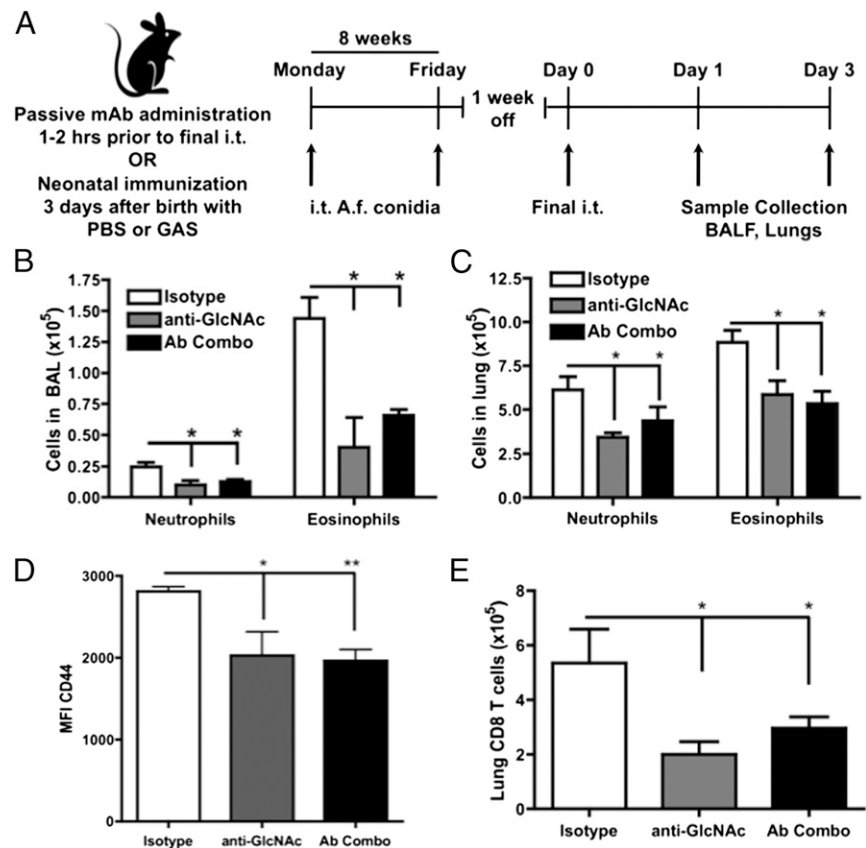
mice with GAS at 3 d of age and rechallenged with GAS as adults. These s.c. immunized mice exhibited an increase in serum levels of anti-GlcNAc IgM similar to mice receiving GAS i.p. as neonates (Supplemental Fig. 3A). These data show that neonatal priming with GAS induces long-lasting effects on serum levels of anti-GlcNAc Ab and resulted in a subsequent dampening of the immune response to chitin and *A. fumigatus* in the lung.

Neonatal GAS immunization dampens the adult AAD induced by A. fumigatus

We next used a similar approach to determine if neonatal immunization with GAS affected the sensitization or provocation of AAD induced by chronic *A. fumigatus* exposure to the lungs. Mice were treated neonatally with PBS or GAS, as described earlier, and then subjected to the chronic *A. fumigatus* sensitization protocol described for Fig. 4A (gating scheme for FACS analysis is shown in Supplemental Fig. 1). Neonatal GAS immunization resulted in the recruitment of fewer total cells, neutrophils, eosinophils, CD4$^+$ T cells, mast cells, and IgE$^+$ B cells in the BAL fluid (Fig. 7A, 7B). Mice receiving s.c. GAS immunization as neonates and subjected to the chronic *A. fumigatus* sensitization protocol described in Fig. 4A also exhibited a decrease in the influx of immune cells into the BAL fluid, similar to mice neonatally immunized i.p. with GAS (Supplemental Fig. 3B–E). In contrast to the dampening of cellular influx to the BAL fluid induced by *A. fumigatus* sensitization after neonatal GAS treatment, immunization of adult mice with GAS prior to an 8-wk *A. fumigatus* sensitization protocol had no effect on the prevention of the allergic response (Supplemental Fig. 4).

Isolates from the digested lung tissue showed similar reductions in these cell types (data not shown). BAL fluid and serum levels of total IgE (Fig. 7C) were found to be lower in the mice immunized neonatally with GAS. Whereas GlcNAc-specific IgG3 was higher

in the BAL fluid after the final challenge with *A. fumigatus* (Fig. 7D, 7E), the decrease in GlcNAc-specific IgM in neonatally immunized GAS-immunized mice may be due to the depletion of IgM by *A. fumigatus* conidia, as GlcNAc-specific IgM prior to sensitization with *A. fumigatus* was higher in the BAL fluid of mice immunized neonatally with GAS than in mice given PBS neonatally. The levels of *A. fumigatus*-specific total Ig in the BAL fluid reactive with soluble *A. fumigatus* allergic extract containing allergens from *A. fumigatus* were lower in mice receiving neonatal GAS (Fig. 7F). Total lung leukocytes were isolated 24 h after final *A. fumigatus* administration and stimulated in vitro with anti-CD3/CD28 in the presence of brefeldin A. The percentage of CD4$^+$IL-4$^+$ cells detected by flow cytometry was lower in GAS-immunized mice compared with PBS-treated mice (Fig. 7G). Total RNA was collected from the lung tissue-derived cells and analyzed by RT-PCR for CCL11, 17, 22, and 24 expression (Fig. 7H), and the BAL fluid (Fig. 7I) and sera (Supplemental Fig. 2D) were analyzed for cytokine expression using the Bio-Plex cytokine assay. Th1 cytokine levels were essentially unchanged, in contrast to the decreased expression of Th2, Th17, and other proinflammatory cytokines, showing that neonatal GAS exposure does not initiate a general immune shift from a Th2 to a Th1 response. To evaluate tissue histology and airway mucus production, paraffin-embedded lung sections from mice neonatally immunized with GAS or treated with PBS after the 8-wk *A. fumigatus* sensitization and 24 h after final *A. fumigatus* administration were stained with H&E (Fig. 7J) and Alcian blue–PAS (Fig. 7K). Mice sensitized and challenged, but not neonatally immunized with GAS, showed marked peribronchovascular inflammation, thickening of the basement membrane and bronchial epithelium, and increased mucus secretion. Alternatively, neonatally GAS-immunized mouse lungs showed minimal signs of remodeling. Our data show that the presence of anti-GlcNAc Abs in the lung, induced by neonatal immunization

FIGURE 5. Anti-*A. fumigatus* Abs decrease the uptake of *A. fumigatus* in vitro and in vivo. (**A**) Representative gating scheme for determining *A. fumigatus*-positive cells. (**B**) BMDMs and (**C**) A549 epithelial cells were cultured with 1×10^5 Alexa 488-labeled *A. fumigatus* conidia, in the presence or absence of an IgM isotype control or an Ab combination (Ab combo) of anti-GlcNAC, anti–sialyllactose-*N*-tetraose, anti–α-1,3 glucan, and *A. fumigatus* uptake was measured by flow cytometry. C57BL/6 mice, treated i.t. with an IgM isotype control or Ab combo, were challenged i.t. with 5×10^5 Alexa 488-labeled *A. fumigatus* conidia, and BAL fluid was collected at 0, 2, and 4 h. (**D**) *A. fumigatus*-positive alveolar macrophages and (**E**) dendritic cells were determined using flow cytometry. Data represent the mean + SEM from three independent experiments with 6 wells or three to five mice per group. Data were analyzed by a two-tailed unpaired *t* test. *$p < 0.05$, **$p < 0.01$.
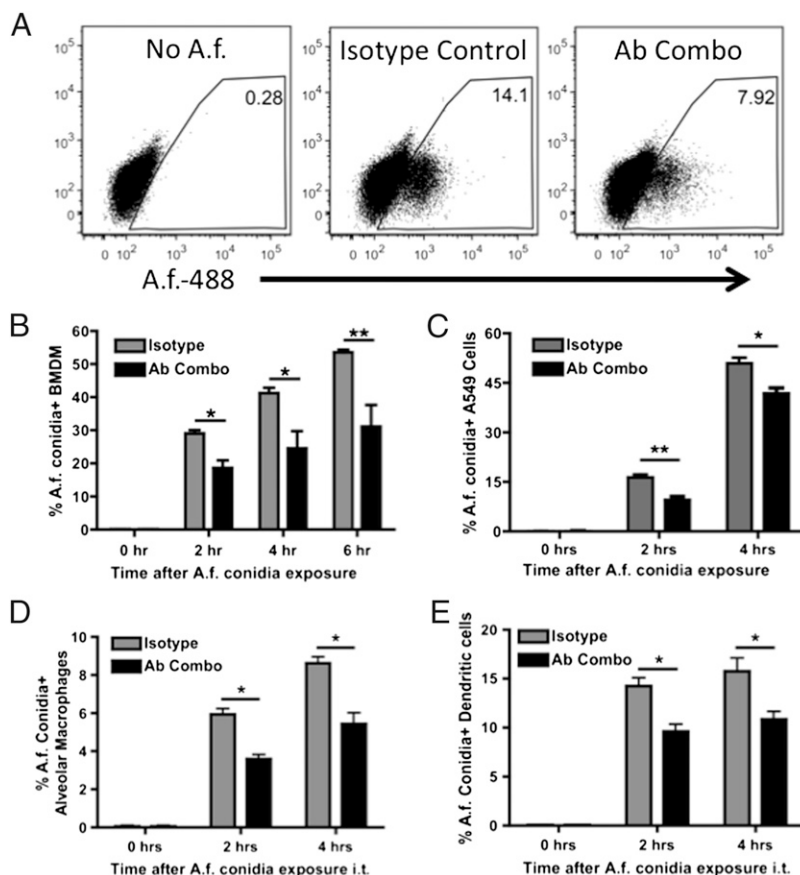
with GAS, is associated with a striking decrease at 18 wk in the development and severity of the AAD induced by chronic *A. fumigatus* exposure. As summarized in Fig. 8, we propose a model by which anti-GlcNAc and similar Abs bind *A. fumigatus* conidia in the lung, preventing them from binding to receptors on resident lung cells as well as immune cells present on the lung. Blockade of these receptor–ligand interactions decreases or diverts uptake of the conidia to other pathways, resulting in a dampening of cytokine and chemokine production as well as decreasing the presentation to and priming of CD4 T cells. In this way, Abs can modulate the development of AAD during its development.

Discussion

There is increasing evidence that primary sensitization to environmental Ags occurs early in life and that the perinatal and early childhood period is considered critical for the development of an appropriate Th1/Th2 balance. However, there is little information regarding how microbial exposure may influence the developing immune system. We found that a single immunization of mice with GAS vaccine during the neonatal period induces anti-GlcNAc Abs that are sustained well into adulthood and protect against the local inflammatory responses induced by chitin and *A. fumigatus* in the lung. In addition, neonatally induced B cells secrete Abs that
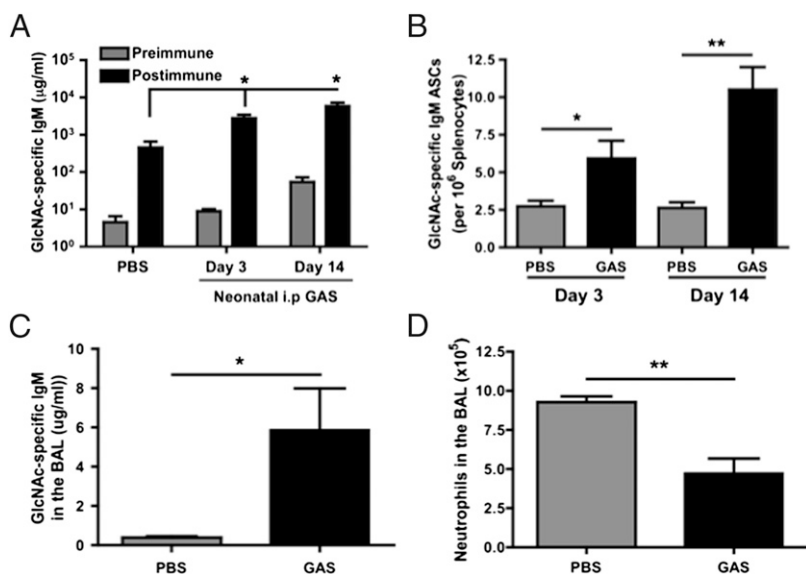

FIGURE 6. Neonatal GAS immunization primes the adult Ab response and dampens the immune response to chitin. Neonatal mice were injected i.p. with PBS or GAS at 3 or 14 d of age. At 8 wk of age, all groups were immunized i.v. with GAS, and sera were collected before (preimmune) and 7 d (postimmune) after reimmunization. (**A**) The level of serum GlcNAc-specific IgM was determined by ELISA. (**B**) ELISPOT analysis was performed 7 d after the neonatal immunizations, day 10 and day 21 respectively, for GlcNAc-specific IgM ASCs. The postimmune mice were challenged i.t. with 100 μg chitin (<10 μm) 7 d after reimmunization at 8 wk, and the BAL fluid level of GlcNAc-specific IgM (**C**) was determined by ELISA and total neutrophil influx (**D**) was determined by flow cytometry 24 h after i.t. challenge. Data represent the mean + SEM from three independent experiments with three to five mice per group. Data were analyzed by a two-tailed unpaired *t* test. *$p < 0.05$, **$p < 0.01$.
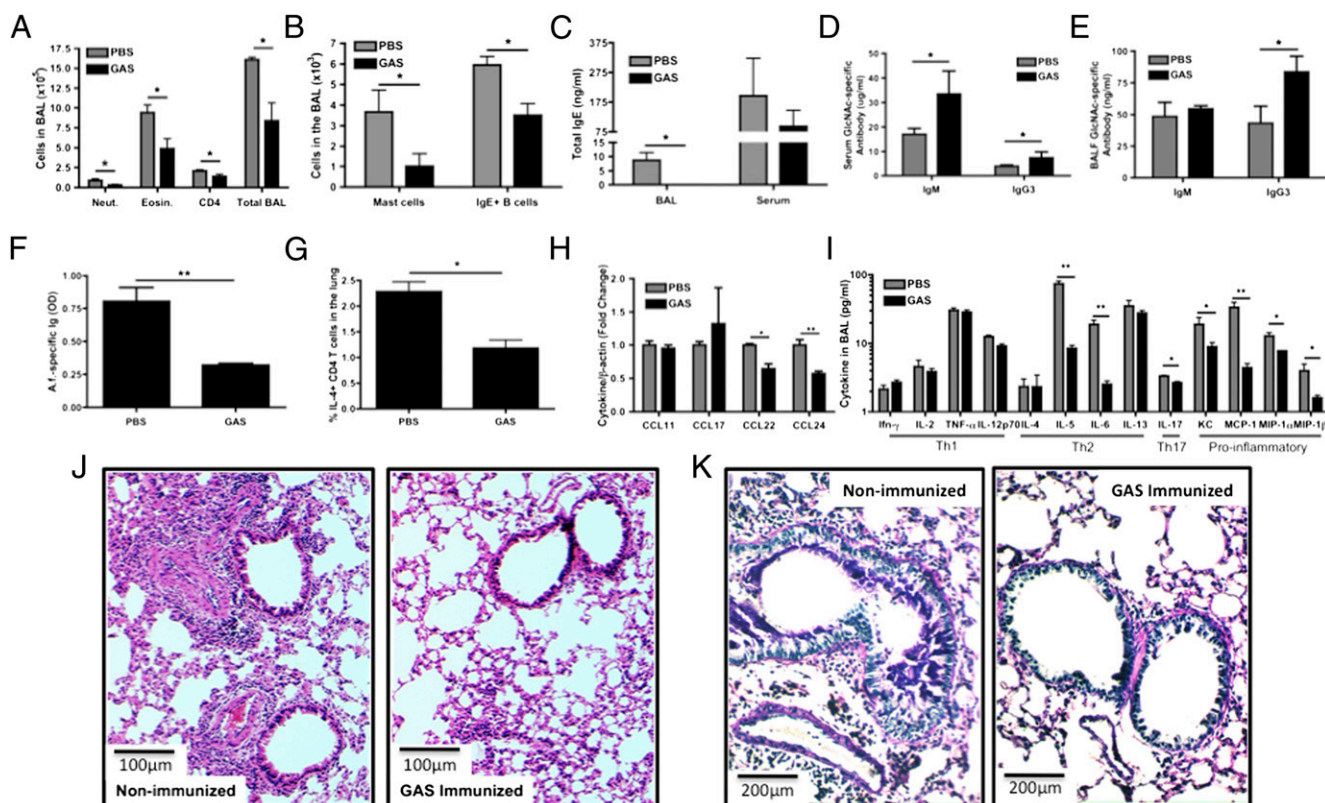
FIGURE 7. Neonatal GAS immunization dampens the adult AAD induced by *A. fumigatus*. Neonatal C57BL/6 mice were immunized once i.p. with PBS or GAS at 3 d of age and grown up to 6–8 wk before starting the chronic sensitization model outlined for Fig. 4A. (**A** and **B**) BAL fluid was collected 3 d after elicitation of the response and analyzed by flow cytometry for (A) neutrophils (neut.), eosinophils (eosin.), CD4$^+$ T cells (CD4), total BAL cells, and (B) mast cells and IgE$^+$ B cells. Sera and BAL fluid were analyzed for (**C**) total IgE, (**D**, **E**) GlcNAc-specific IgM and IgG3, and (**F**) *A. fumigatus*-specific total Ig against purified *A. fumigatus* allergic extract using ELISA. (**G**) Total lung leukocytes were stimulated with anti-CD3/CD28 in the presence of brefeldin A, and the percentage of CD4$^+$IL-4$^+$ cells was determined by flow cytometry. (**H**) Total RNA was collected from lung-derived cells and analyzed by RT-PCR for CCL11, 17, 22, and 24 expression. (**I**) BAL fluid was analyzed for cytokine expression using the Bio-Plex cytokine assay. Data were analyzed by an unpaired *t* test. *$p < 0.05$, **$p < 0.01$. See also Supplemental Figs. 1 and 2. Paraffin-embedded lung sections from PBS- and GAS-immunized neonatal mice were stained with (**J**) H&E and (**K**) Alcian blue–PAS. Data represent the mean ± SEM from three independent experiments with three to five mice per group.

decrease the allergic response to *A. fumigatus*. Our results suggest that Ag exposure during the neonatal period, whether derived from environmental sources, self-antigens, or vaccination, has dramatic effects on the adult Ab response and attenuates the development of AAD.

A highly significant negative correlation between GAS infections (scarlet fever) and asthma was found by annual, seasonal (by month), and geographic (by state) comparisons (32). Another study found that a strong inverse relationship of allergic asthma, but not atopy, was associated with concentrations of peptidoglycan-associated muramic acid in mattress dust (33). The cell wall of Gram-positive bacteria, such as GAS, contain large amounts of peptidoglycan, a biopolymer consisting of alternating units of GlcNAc and *N*-acetylmuramic acid. Our data are in agreement with these studies as we found that neonatal immunization with GAS dampened the development and severity of AAD induced by *A. fumigatus* in mice. Multiple studies suggested that the timing between infection and allergen exposure is critical as to whether protection or disease ensues in humans (34). In our previous publications, we have shown that neonatal exposure to bacteria expressing phosphorylcholine and α-1,3 glucans results in permanent and distinct alterations in the H and L chain composition of B cells with these specificities in the adult repertoire (35). Similar changes occur in the repertoire of B cell clones involved in the anti-GlcNAc response to GAS after neonatal GAS versus adult immunization. (B.L.P. Dizon and J.F. Kearney, submitted for

publication). Furthermore, the timing of neonatal GAS immunization, day 3 versus day 14, resulted in a significant difference in the baseline levels and recall responses of anti-GlcNAc Abs. In agreement with these findings, our current results show that the timing of Ag or microbial exposure is critical in influencing the development of Ab-producing B cell clones that aid in the prevention of AAD. In contrast to the protective effect of neonatal immunization with GAS on *A. fumigatus* sensitization, immunization of adult mice with GAS prior to the 8-wk *A. fumigatus* sensitization protocol had no effect on the prevention of the allergic response (Supplemental Fig. 4), even though the anti-GlcNAc Abs at the time of initiation of the sensitization protocol are similar to those described in Fig. 6A (PBS group).

Numerous studies have shown that Ab responses to both T-dependent Ags and purified PSs in general are poor in the neonatal period, findings partially attributed to a deficit in bone marrow stromal support of ASCs (36). However, certain B cell clones are generated only during fetal and neonatal life and remain as permanent members of the adult repertoire (37). We observed that neonatal immunization has long-lasting effects on the adult B cell Ab repertoire. These data show that systemic neonatal exposure to GAS produces a long-lived adult IgM response to GlcNAc in mice, which plays a protective role against lung sensitization to chitin and *A. fumigatus*. Young infants and children rarely get significant GAS infection until age 3, but it is a very frequent cause of illness in school-age children. These observations cor-
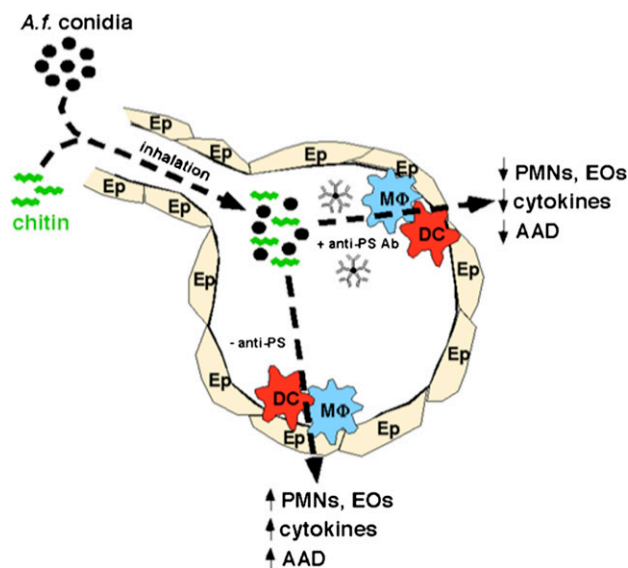
FIGURE 8. Model for the prevention of AAD development by anti-PS Abs. This schematic shows our proposed model for the protection against *A. fumigatus*-induced AAD.

relate with experiments showing that normal GAS-uninfected children <4 y old in the United States have undetectable levels of specific anti-GlcNAc Abs perhaps because the carrier rate of *S. pyogenes* in healthy populations is only 5%. These very low levels of GlcNAc-inhibitable, anti-group A reactive Ab gradually increase as the children get older (38). It will be of interest to determine through retrospective analyses possible correlations between infant levels of anti-GlcNAc Ab and subsequent development of AAD. Our model has concentrated on GAS to generate anti-GlcNAc Abs in our mouse model; however, GlcNAc-containing Ags are expressed by many other organisms including *Staphylococcus aureus*, *Escherichia coli* (39), *Listeria monocytogenes* (40), fungi, and helminths. We have shown that these organisms generate GlcNAc-specific Abs in mice raising the likelihood that infections or carriage of these and other similar microorganisms may also be associated with protection against AAD.

If such protective Abs are produced by deliberate immunization with bacteria, why are similar Abs not produced to the same *A. fumigatus* epitopes during the sensitization period? We and others have found that deliberate immunization of adult mice with chitin or *A. fumigatus* induces very little Ab reactive with fungal cell wall PS components [(11) and data not shown], nor in humans infected with *A. fumigatus* are humoral responses elicited (41, 42). These findings contrast with the comparatively high and long-lived anti-PS responses produced after neonatal immunization and rechallenge with the bacterial vaccines. This may be due to the way the *A. fumigatus* polymeric cell wall Ags are presented or to a lack of crucial costimulatory molecules similar to those associated with bacteria or immuno-inhibitory molecules produced by *A. fumigatus* (43, 44). In our model, mice developed Abs against soluble allergic Ags found in the *A. fumigatus* allergic extract used in clinical settings. GAS-immunized mice had significantly lower Ab responses to the allergic extract than those of PBS-treated mice. These results suggest that the anti-GlcNAc Abs are clearing or shielding the *A. fumigatus* from the immune system resulting in lower Ab responses to soluble protein Ags.

The mechanisms by which Abs regulate biology and host defense are diverse and can include neutralization, Ab-dependent cell-mediated cytotoxicity, opsonization, and Ag presentation (reviewed

in Ref. 45), in addition to other mechanisms that are only beginning to be appreciated (46). IgG Abs have been studied in great detail, whereas aspects of the induction and role of natural IgM Abs have been studied less extensively. One of the reasons that the functional significance of IgM is often ignored is the assumption that insufficient concentrations exist at the site of Ag exposure. B1 cells in the peritoneal and pleural cavities and MZ B cells in the spleen give rise primarily to IgM isotype-secreting cells (47, 48), and recent reports show that IgM plays underappreciated protective roles in a variety of microbial infections (49–52). Although the alveolar epithelia are associated with mostly plasma-derived monomeric IgA and IgG, there is low but detectable IgM (53–55). Furthermore, a recent study suggests that IgM promotes macrophage-mediated clearance of Ag in a size-dependent manner (56), specifically promoting clearance of small-size (<3 μm) particles, which are smaller than swollen conidia and our chitin particles (4–10 μm). Our results are in agreement with these findings as we observe that IgM fails to promote the uptake of chitin and *A. fumigatus* by macrophages, dendritic cells, and epithelial cells, actually inhibiting uptake by 25–50%. Natural IgM Abs have been shown to play a role in mediating immunity against *Pneumocystis murina* (57), and our data indicate that anti-GlcNAc IgM Abs dampen the binding or internalization of chitin and *A. fumigatus*. Taken together, the decreased uptake of Ag and subsequent sensitization to *A. fumigatus* in our chronic AAD model suggests that anti-PS IgM alters the recognition, activation, and immune response against *A. fumigatus*. As we show, IgG3 is consistently observed in lung fluids and sera of GAS-immunized mice. Mice immunized with natural bacterial (not conjugated) carbohydrate polymers consistently produce largely IgM and to a lesser extent IgG3 isotype and some IgA in serum. These types of T-independent Ab responses do not normally induce the other IgG isotypes that have the potential to bind via complexes to trigger well-characterized IgG FcγRs. In contrast, IgG3 Abs do not appear to bind known IgG Fc receptors (58). Although it has not yet been shown conclusively, IgG3 complexes, in contrast to other IgG isotypes (59), may also give different signals to APCs and in this way dampen their cytokine responses and Ag-presenting capabilities, similar to those properties proposed for IgM and the human IgG3 equivalent (IgG2), as discussed for other model systems (60).

Sensitization in many mouse asthma/allergy studies is achieved by using alum adjuvant-based models with highly purified allergens or surrogate sensitizing Ags administered systemically (61). However, because sensitization by allergen-bearing organisms involved in human asthma occurs via airways or skin after chronic exposure, sensitization through these portals constitutes a more relevant mouse model. Moreover, alum-based models have proved ineffective in evaluating interventional procedures to control the development of allergic disease (62, 63), which in many cases occurs over a period of time by incremental exposure to sensitizing allergens. It is clear that the immune responses in the lung to particulate conidia versus purified or recombinant *A. fumigatus* allergens are significantly different (64–66). A recent review chronicles the drawbacks of these models and emphasizes the need for better models that more closely mimic the development and progression of human AAD (5). Our use of a chronic low-dose model for induction of AAD, using live *A. fumigatus* conidia without artificial adjuvants, has revealed that increased natural Abs through neonatal vaccination elicits significant protection against pulmonary challenge with *A. fumigatus*.

In mice, carbohydrates and carbohydrate-modified proteins induce multiple types of innate immune responses by ligating a variety of selectins, TLRs, CR3 or MAC1, mannose receptor, and dectin-1. For example, β-glucans expressed on *A. fumigatus* pro-

mote strong inflammatory cytokine production (67–69), and it has been proposed that α-1,3 glucans shield dectin-1 from binding to β-glucans in *Histoplasma capsulatum* (70). In a similar fashion, anti-PS Abs binding *A. fumigatus* may shield a variety of innate receptor–ligand interactions and lower the levels of asthma-associated cytokines and chemokines produced. Our current results support this hypothesis and show that levels of IL-5, IL-6, MCP-1, MIP-1α/β, IL-17, CCL17, CCL22, and CCL24, all of which play roles in AAD (71), are decreased in the presence of anti-PS Abs. Such receptors expressed on macrophages and dendritic cells are also involved in the clearance or intracellular fate of microorganisms (72) and Ag processing and presentation pathways (41, 73). However, there are conflicting data within the literature suggesting that chitin is associated with both enhancement (6, 10) and inhibition (74, 75) of AAD. We consider that naturally occurring chitin particles and other conserved cell wall components act as immunogenic carriers, not as allergens themselves, with the potential to bring protein allergens into cells as "cargo." These carbohydrates may play a role in regulating the presentation of Ags/allergens to T cells (76–78). Our data support the idea that Abs to these conserved structures play a role in diverting their allergenic "cargo" into pathways less conducive to T cell sensitization. Taken together, the results from our model show that natural Abs from B cells generated and primed during neonatal bacterial exposure play a role in altering innate receptor–ligand interactions, subsequent inflammatory responses, and Ag processing and presentation.

The development of asthmatic allergic disease is a long and complex process related to the development of a specific phenotype, environmental factors, and genetic susceptibility. As a result, it is highly unlikely that there will be a single mechanistic explanation for the induction, maintenance, or especially prevention of such diseases. Nevertheless, our data clearly show that the timing of Ag exposure during the neonatal period has long-lasting effects on the baseline natural Ab levels and recall responses as an adult. We show that the Ab responses induced by conserved bacterial PS Ags have dampening effects on the development and severity of *A. fumigatus*-induced AAD by downregulating both the influx of allergen-associated cell types and multiple cytokines and chemokines associated with AAD. The ability to alter drastically the magnitude and specificity of an Ab response, through the timing of neonatal exposure, is an important observation and may lead to vaccination or probiotic protocols during early life that will dampen the development of airway disease. Exposure of neonates to microbial stimulation may shift the Th1/Th2 cytokine balance, but it may also involve changes in the magnitude and repertoire of the responding Abs, a property that may be exploited for therapeutic intervention.

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Disclosures

The authors have no financial conflicts of interest.

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